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CC ligand 2 levels are increased in LPS-stimulated peripheral monocytes of patients with non-small cell lung cancer ☆

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Summary
Non-small cell lung cancer (NSCLC) shows a particular aggressive behaviour. Tumour associated macrophages (TAMs) play an important role in tumour growth and progression and CC ligand 2 (CCL2)/CCR2 axis is markedly involved in their recruitment in the tumour mass from the circulation.
The aim of this study was to determine the plasma levels of CCL2 and the expression of CCR2 in the peripheral blood mononuclear cells (PBMCs) of 18 smokers with NSCLC, eight healthy smokers and nine non-smokers. Then, we investigated CCL2 levels in the supernatants of unstimulated and LPS-stimulated PBMC cultures of the same groups of patients.
CCL2 levels in plasma and supernatants of PBMC cultures were determined by ELISA. CCR2 expression in PBMC cytospins was assessed by immunocytochemistry.
CCL2 plasma levels and CCR2 expression by PBMCs were similar in patients with NSCLC, healthy smokers and non-smokers. In the supernatants of unstimulated PBMC cultures, CCL2 content was not different between the three groups of subjects. Supernatants of LPS-stimulated PBMCs of NSCLC patients showed a higher content of CCL2 as compared to supernatants of non-smokers ($p<0.005$). CCL2 content increased 28.5-fold vs baseline production in the group of NSCLC patients, 15-fold in healthy smokers and 13-fold in the group of non-smokers.

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In conclusion, after LPS stimulation, PBMCs of patients with NSCLC release higher levels of CCL2 as compared to those of non-smokers, supporting the hypothesis of a CCL2 involvement in NSCLC biology.

Introduction

The overall 5-year survival rate of patients with non-small cell lung cancer (NSCLC) is less than 15%.[1] The complex interaction between tumour cells and the host immune network contributes to determine the aggressive behaviour of lung cancer.[2]

Cells belonging to the monocyte-macrophages lineage are a major component of the leucocyte infiltrate of neoplasms and tumour associated macrophages (TAMs) are recognized to play an important role in tumour growth and progression.[3]

It is generally accepted that most TAMs are derived from peripheral blood monocytes recruited into the tumour mass from the circulation.[3,4] Recruitment of circulating monocytes into the tumour mass is directed by chemokines. Chemokines are small heparin-binding proteins playing a crucial role in directing the movement of mononuclear cells throughout the body, engendering the adaptive immune response and contributing to the pathogenesis of a variety of diseases.[5] They are divided into four families on the basis of differences in structure and function. Monocyte chemotactic protein-1 is the most thoroughly characterized member of the CC chemokine family and it has been termed as CC ligand 2 (CCL2) in the new classification system.[5,6] It mainly acts through the CCR2 receptor which is expressed by monocytes, immature dendritic cells and memory T cells.[5]

CCL2 stimulates chemotaxis of peripheral blood monocytes and of memory T cells.[5,7] It also induces calcium flux, respiratory burst activity, adhesion molecule and proinflammatory cytokine expression in monocytes.[8,9] Many evidences support the hypothesis that CCL2 regulates tumour growth and progression by recruiting inflammatory cells into the tumour mass, enhancing the cytotoxic activity in monocytes and natural killer cells and modulating the angiogenesis and the immune response against the tumour through the regulation of mediator release by monocytes.[10–15]

Different tumour cells express CCL2 both *in vivo* and *in vitro*, including NSCLC cells.[2,10–12,15,16] CCL2 is also expressed by TAMs and activated human peripheral blood monocytes.[17–19]

The aims of this study were to determine the plasma levels of CCL2 and the expression of CCR2 in peripheral blood mononuclear cells (PBMCs) of smokers with NSCLC, healthy smokers and non-smokers, and to investigate CCL2 levels in the supernatants of unstimulated and LPS-stimulated PBMC culture of the same groups of patients.

Materials and methods

Study population

The study group comprised 18 patients with primary NSCLC, and 17 control subjects (nine smokers and eight non-smokers). All subjects underwent an interview and data collected included age, gender, occupation, and smoking history. Routine pulmonary function tests were performed and baseline FEV_1 and FEV_1/FVC were recorded. Lung cancer patients had not received chemotherapy or radiation before surgery. In these patients, histopathological diagnosis was done in samples obtained at biopsy (10 of the 18) or at surgery (8 of the 18). The pathologic tumour stage (p stage) was determined according to the revised TNM classification.[20]

Blood samples and PBMC isolation

Peripheral blood samples from patients and controls were collected into Vacutainer® sterile tubes (BD, Franklin Lakes, NJ, USA) containing 2500 U Liquemin® (La Roche A.G, Wyhlen, Germany). Lung cancer patients were bleeded the day of biopsy or surgery. Samples were diluted 1:1 with RPMI-1640 medium and layered on Hystopaque-1077® (Sigma, Milan, Italy). Samples were centrifuged three times and each time the interface band of PBMCs was collected and resuspended in cold PBS (phosphate buffered saline). After the last centrifugation, cells were resuspended in RPMI-1640 medium (BE 12-167F, Biowhittaker™, Cambrex Bioscience, Verviers, Belgium) containing 10% heat-inactivated FCS (CHA 2111D Celbio, Milan, Italy), 1% L-glutammin (BE 17-605E, Biowhittaker™, Cambrex Bioscience, Verviers, Belgium), and 0.25% antibiotics (penicillin–streptomycin mixture, 17-602E Biowhittaker™, Cambrex Bioscience, Verviers, Belgium). Plasma samples were collected and stored at −20 °C.

Isolation of adherent cells

Isolated PBMCs from subjects were cultured 1×10^6 cells/ml medium in 24 well tissue culture plates. Cells were incubated for 1 h 30 min at 37 °C in humified atmosphere, and non-adherent cells were removed by washing with PBS. Monocytes were incubated in RPMI-1640 with or without 10 μg/ml LPS (2654, Sigma, Milan, Italy). After 24, 48 or 72 h supernatants were withdrawn, centrifuged and stored at −20 °C.

On the basis of preliminary experiments, we decided to assess CCL2 levels in the supernatants of PBMCs incubated for 48 h with or without LPS.

CCL2 quantification

CCL2 levels in the plasma and supernatants were determined by ELISA (CYT20, Chemicon international Inc., Temecula, CA, USA). The assay was performed according to the manufacturer's instructions. The minimal detectable level was 13.1 pg/ml.

Immunocytochemistry

After PBMC separation, 4×10^6 cells were withdrawn and cytospinned at a concentration of 1×10^6 cells/ml. Differential cell count was performed after Diff-Quick staining of slides. Immunocytochemistry was performed by using the method of the streptavidin biotin complex conjugated with alkaline phosphatase. Sections were incubated with a goat anti-human polyclonal antibody anti-CCR2 (sc-6228, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) 1:50 in TMS (tris maleate saponin solution). Slides were subsequently incubated with rabbit anti-goat biotinylated immunoglobulins (E466, Dako Ltd., Glostrup, Denmark) and with streptavidin biotin complex conjugated to alkaline phosphatase (StreptABComplex/AP, K391, Dako Ltd., Glostrup, Denmark). Immunoreactivity was visualized with fast red (K699; Dako Ltd.). Sections were counterstained with haematoxylin and mounted in Glycergel (C563; Dako Ltd.).

Cell count was performed using an Olympus BX41 light microscope (Olympus Optical Co., Hamburg, Germany). For each slide, at least 400 monocytes were counted in at least 10 randomly selected fields. Monocytes were identified by means of morphologic criteria and results were expressed as percentage of CCR2+ve monocytes/total number of monocytes. Fourteen cytopsins of blood mononuclear cells of patients with NSCLC, six of healthy smokers and eight of non-smokers had a satisfactory quality to allow the determination of the CCR2+ve mononuclear cells percentage.

Statistical analysis

Analysis of variance (ANOVA) followed by unpaired *t*-test for clinical data and the non-parametric Kruskall–Wallis test followed by the Mann–Whitney *U* test for histological data were used to determine differences between groups. The chemokine content in unstimulated and LPS-stimulated cell culture supernatants of the same groups of subjects was compared by the Wilcoxon signed rank test.

Results

Subjects

Patient's characteristics are summarized in Table 1. All healthy smokers were current smokers except one who quit smoking 2 years before entering into the study. In the group of non-smokers, one subject was an ex-smoker from 25 years (0.6 pack-years) whereas the remaining seven were life long non-smokers. The mean age was similar in the three groups of subjects. The two groups of smokers were similar with regard to number of pack-years. Smokers with lung cancer had a lower FEV_1 (% predicted) and a lower FEV_1/FVC ratio (%) than healthy smokers ($p<0.05$). Their FEV_1 was also lower as compared to non-smokers ($p<0.05$). Histological diagnosis revealed that eight patients had squamous cell carcinoma (44%), seven had adenocarcinoma (39%) and three had undifferentiated NSCLC (17%). Eight patients (44%) were classified as stage I disease, and the remaining 10 (56%) as stages II, III and IV disease (late stage).

Table 1 Demographic and clinical data.

	Smokers with lung cancer ($n=18$)	Healthy smokers ($n=9$)	Non-smokers ($n=8$)
Sex (male/female)	18/0	8/1	7/1
Age (yrs)	65±2	60±3	57±2
Pack-years	52±6**	37±4**	0.1±0.1
FEV_1 (% pred.)	74±7*,***	97±4	102±7
FEV_1/FVC (%)	69±5***	87±4	80±3
Type of tumour (no. of subjects)			
Adenocarcinoma	7		
Squamous cell carcinoma	8		
Undifferentiated	3		
TNM stage (no. of subjects)			
I	8		
II	2		
III	3		
IV	5		

Data are presented as mean±standard error of the mean; *$p<0.05$ and **$p<0.001$ vs non-smokers; ***$p<0.05$ vs healthy smokers. FEV_1 and FEV_1/FVC were measured in 15 patients with lung cancer.

CCL2 and CCR2 quantification

The CCL2 content in plasma samples was similar in smokers with lung cancer, healthy smokers and non-smokers [median (interquartile range): 44.0 (25.9–69.2) vs 63.6 (18.8–77.6) vs 16.6 (11.8–33.4) pg/ml, respectively].

Similarly, the CCR2 expression was comparable in PBMC cytospins of smokers with lung cancer, healthy smokers and non-smokers [median (interquartile range): 44.7% (27.8–56.0) vs 35.9% (24.8–39.0) vs 31.7% (27.5–41.8), respectively].

In the supernatants of unstimulated PBMC cultures, the CCL2 content was not different among the three groups of subjects (Fig. 1A). Conversely, supernatants of LPS-stimulated PBMC cultures of smokers with lung cancer showed a higher content of CCL2 than those of non-smokers [median (interquartile range): 2023.4 (1008.9–2906.3) vs 660.0 (276.6–741.1) pg/ml respectively, $p<0.005$]. On the other hand, no significant differences were found with healthy smokers [2023.4 (1008.9–2906.3) vs 1212.5 (480.5–1691.0) pg/ml, respectively] (Fig. 1B).

In the supernatants of smokers with lung cancer, LPS stimulation produced a 28.5-fold increase in the CCL2 production compared to unstimulated PBMC cultures [(2023.4 (1008.9–2906.3) vs 70.9 (36.9–168.9) pg/ml, respectively, $p<0.005$] (Fig. 2). In the supernatants of healthy smokers, LPS produced a 15-fold increase [1212.5 (480.5–1691.0) vs 82.3 (13.8–243.7) pg/ml, respectively, $p<0.05$]. Finally, in the group of non-smokers, a 13-fold increase was observed [660.0 (276.6–741.0) vs 49.0 (24.2–114.4) pg/ml, respectively, $p<0.05$] (Fig. 2).

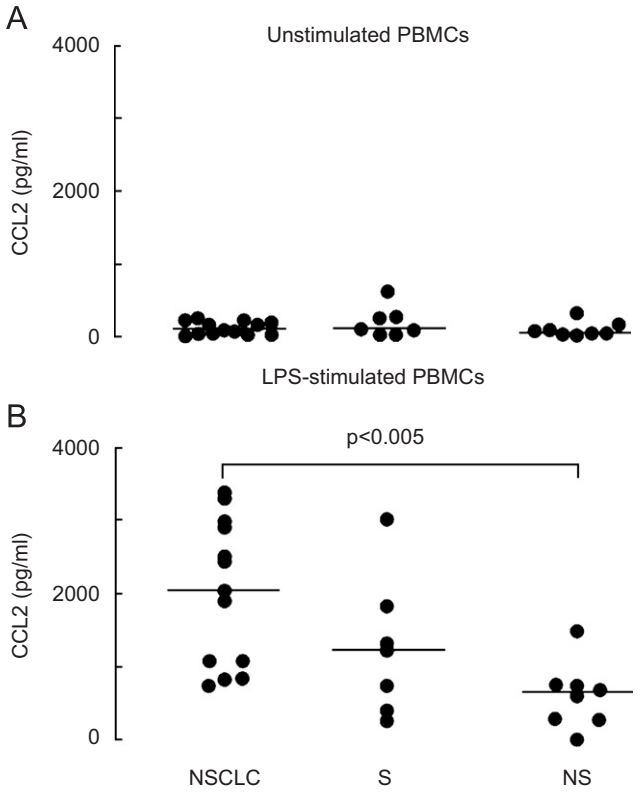

Figure 1 Individual values of MCP-1 levels in the supernatants of: (A) unstimulated and (B) LPS-stimulated PBMC cells cultures of smokers with non-small cell lung cancer (NSCLC), healthy smokers (S) and non-smokers (NS). Horizontal bar represents median values. CCL2 levels were measured by ELISA. NSCLC: $n = 13$; S: $n = 7$; NS: $n = 8$.

The CCL2 content in plasma was similar in patients with stage I disease as compared to patients with late stage disease [34.1 (20.6–64.3) vs 48.2 (30.3–69.4) pg/ml, respectively]. Similarly, the CCR2 expression was not different in PBMC cytospins of patients with stage I disease as compared to patients with late stage disease [42.9 (38.4–57.3) vs 44.7 (27.2–52.0) pg/ml, respectively].

Discussion

In the present study, similar levels of CCL2 in plasma of smokers with NSCLC, healthy smokers and non-smokers were found. Similarly, the percentage of CCR2+ve monocytes was not different in PBMC cytospins of the three studied groups. CCL2 content in plasma and CCR-2 expression in PBMC cytospins were similar in patients with early stage lung cancer as compared to those with late stage lung cancer.

CCL2 is believed to be involved in the recruitment of circulating monocytes to the tumour site.[2,11,17,21,22] Indeed, CCL2 levels in breast cancer homogenates and CCL2 mRNA in gastric carcinoma and esophageal squamous cell carcinoma correlate with TAMs accumulation in the tumours.[17,21,22] Arenberg et al.[2] have shown that the level of CCL2 in NSCLC homogenates was higher than in normal lung tissue and correlated with TAMs infiltration.

We observed similar CCL2 plasma levels in patients with NSCLC compared to controls. However, our findings do not exclude a role of CCL2/CCR2 axis in the recruitment of monocytes to the lung tumour, since it is likely that the CCL2 plasma levels do not exactly mirror the chemokine production in lung cancer tissue.

The CCR2 is the only known CCL2 receptor and it is expressed by monocytes. Recently, two functional subsets of murine blood monocytes have been identified and it has been suggested that only the subset corresponding to the human monocyte population known as $CD14^{+}CD62L^{+}CCR2^{+}$ bears the CCR2 and it is actively recruited to inflamed tissues.[23]

Although CCR2 binds to other chemokines, the CCL2/CCR2 axis has unique effects on mononuclear cell migration and it is likely an important regulator in inflammatory diseases. In murine models of atherosclerosis and in experimental allergic encephalomyelitis, the CCR2 deficiency reduced macrophage recruitment to the site of disease and the severity of disease itself.[24] CCR2 expression has been found on tumour infiltrating leukocytes in ovarian, nasopharyngeal and esophageal squamous cell carcinomas, supporting its involvement in the process of inflammatory cells recruitment to the cancer site.[22,25,26]

Our finding of a similar percentage of CCR2+ve mononuclear cells in the PBMC cytospins of patients with NSCLC, healthy smokers and healthy controls does not rule out the possibility that CCR2 has a key role in the regulation of the process of macrophage accumulation to the tumour site. In fact, Katschke et al.[27] have demonstrated a differential expression of chemokine receptors on peripheral blood, synovial fluid and synovial tissue monocytes/macrophages in rheumatoid arthritis. Patients with rheumatoid arthritis showed a similar CCR2 expression in PBMCs, but a lower percentage of CCR2+ve monocytes in the synovial fluid compared to normal subjects. The authors concluded that, in rheumatoid arthritis, CCR2 is mainly involved in the tissue retention of recruited macrophages rather than in monocyte recruitment from the circulation.[27]

Further studies are necessary to clarify the possible differential roles of CCR2 expression by monocytes/macrophages in NSCLC.

The absence of difference between early and late stage NSCLC with regard to CCL2 plasma levels and PBMC CCR2 expression may result from a mismatch in the compartments of blood and lung cancer tissue. Other explanations include the possibility that the current study was underpowered to demonstrate significant differences in patients with different tumour stages.

The major finding of our study is that LPS-stimulated PBMC cultures of patients with NSCLC produced higher levels of CCL2 than PBMC cultures of non-smokers. Moreover, after LPS stimulation, the CCL2 increase vs baseline production was greater in patients with NSCLC as compared to controls. Taken together, these data would suggest that peripheral monocytes of patients with NSCLC exhibit a greater response to inflammatory stimuli in terms of CCL2 production. Therefore, once recruited to the tumour site, this monocyte population might contribute to enhance the local levels of CCL2 release.

Many evidences suggest that chemokines behave more than attractants, due to their pleiotropic effects that

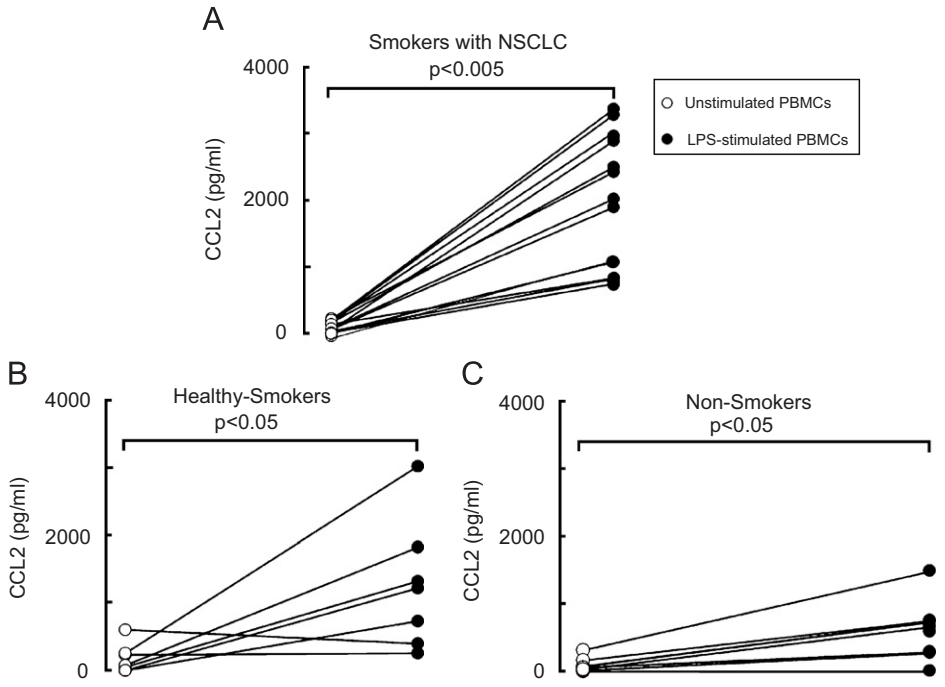

Figure 2 LPS-stimulated release of MCP-1 in the supernatants of patients with non-small cell lung cancer (NSCLC), healthy smokers and non-smokers compared to the release in unstimulated supernatants. PBMC cell cultures were stimulated with 10 μg/ml LPS for 48 h. CCL2 levels were measured by ELISA. NSCLC: $n = 13$; S: $n = 7$; NS: $n = 8$.

impact the cancer pathobiology in a way that depends on the particular setting in which they are expressed.[24] Animal models and *in vitro* studies indicate that CCL2 can enhance macrophage cytolitic activity against tumour cells, stimulates NK cells cytotoxic activity and suppresses systemic spread of human lung adenocarcinoma cells by a NK cell-mediated mechanism.[13,24,28] On the other hand, CCL2 can induce angiogenesis. Indeed, the local expression of CCL2 correlates with the levels of potent angiogenic factors [thymidine phosphorilase (TP), vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF)-α, interleukin (IL)-8] in human breast cancer extracts and CCL2+ve samples of carcinoma of the esophagus show higher TP expression and microvessels density than those CCL2 negative.[17,29] Finally, in human prostate cancer and in carcinoma of the esophagus, CCL2 would promote tumour cell proliferation, while in pancreatic cancer, circulating CCL2 levels inversely correlate with the tumour proliferative activity.[10,12,29]

The functional significance of CCL2 expression in human NCSLC remains to be clarified. However, the finding of an increased chemokine expression at tumour tissue level compared to normal lung and the correlation between CCL2 expression and macrophage infiltration suggest that CCL2 may be involved in the NSCLC biology.[2] Our findings are coherent with these results and indicate that, a local recruitment of monocytes, prone to release high levels of CCL2, would contribute to modify the tumour microenvironment, thus modulating inflammatory cell activation and chemotaxis, angiogenesis and, eventually, tumour cell proliferation.

In conclusion, the current authors have found that, after LPS stimulation, PBMCs of patients with NSCLC release higher levels of CCL2 as compared to those of non-smokers. These results suggest the CCL2 involvement in NSCLC biology. Future investigations will be needed to better elucidate the functional role of CCL2 in NSCLC growth and progression.

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